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Resveratrol-induced apoptosis is enhanced in acute lymphoblastic leukemia cells by modulation of the mitochondrial permeability transition pore

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Abstract

We have previously shown that resveratrol can induce apoptotic cell death in cell lines established from patients with acute lymphoblastic leukemia (ALL). Cyclosporin A (CsA) and PK11195 are modulators of the mitochondrial permeability transition pore (MPTP) which has been proposed to play a critical role in regulating survival and death. Using SEM and RS4;11 lines with the t(4;11) translocation, the B-ALL line REH, and the T-ALL line Jurkat, we show that pre-treatment with CsA or PK11195 significantly enhances resveratrol-mediated apoptosis and mitochondrial membrane depolarization in these cells, as measured by annexin V and JC-1 staining, respectively. No significant multi-drug resistance efflux of the fluorescent substrate calcein was observed in these ALL lines, indicating that CsA and PK11195 were acting at the level of the mitochondria to enhance loss of mitochondrial membrane potential and induction of apoptosis. These data suggest targeting the MPTP sensitizes B- and T-cell ALL to the anti-cancer activity of resveratrol, and may be particularly useful for the treatment of high-risk t(4;11) ALL. Published by Elsevier Ireland Ltd.

Keywords: Leukemia; Apoptosis; Resveratrol; Cyclosporin A; PK11195

1. Introduction

Resveratrol (3,4′,5 -trihydroxy-*trans*-stilbene) is a member of the phytoalexin class of antibiotics and is induced in a variety of plants in response to fungal infection, environmental stress, or injury. Resveratrol is a well-known component of the skin of grapes, is present in high concentrations in red wine, and is also found in other fruits such as blueberries, mulberries, rhubarb, and cranberries. Jang et al. [1] was first to describe the ability of resveratrol to inhibit events associated with initiation, promotion, and progression of cancer. Resveratrol has been shown to inhibit the proliferation and/or induce apoptotic cell death in a number of different types of cancer cells in vitro. Mechanistic studies have revealed that resveratrol can act as an antioxidant, inhibit transcription factor activation, inhibit kinase pathways involved in cell signaling, as well as those involved in progression of the cell cycle (reviewed in Ref. [2]). Resveratrol has also been shown to inhibit the F1 complex of the F0/F1 ATPase proton pump in the inner mitochondrial

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membrane [3] that is responsible for the synthesis of ATP from ADP during oxidative phosphorylation. Disruption of the proton pumping appears to be at least partly responsible for resveratrol's ability to induce depolarization of mitochondrial membranes [4,5].

Mitochondrial membrane potential ($\Delta\Psi mt$) tightly regulates opening of the mitochondrial permeability transition pore (MPTP), which is a central event during mitochondria-mediated apoptosis [6]. The MPTP is a multi-protein complex formed between the inner and outer mitochondrial membranes. Seven proteins have been implicated in pore formation, including the adenine nucleotide translocase (ANT) located in the inner membrane and the voltage-dependent anion channel (VDAC) located in the outer membrane [7]. Other proteins that appear to be a part of this complex are cyclophilin D found in the mitochondrial matrix, which binds to the ANT, creatine kinase in the intermembrane space, and hexokinase in the cytosol. Recently, the peripheral benzodiazepine receptor (PBR) localized in the outer mitochondrial membrane has also been associated with the regulation of MPTP formation [7]. The MPTP helps regulate matrix Ca^{2+} concentration, pH, and $\Delta\Psi mt$ by acting as a conductance channel and transient opening of the pore helps to maintain the electrochemical gradient that drives ATP synthesis.

Chromosomal abnormalities in the ALL-1 gene (also known as MLL, HRX, and HTRX1) on chromosome 11 are frequently involved in childhood ALL. The chromosomal translocation t(4;11)(q21;q23) is found in greater than 60% of infants, 2% of children, and 3–6% of adults diagnosed with ALL and the presence of this chromosomal abnormality is strongly associated with an exceedingly poor prognosis [8]. More recently, the frequency of the t(4;11) translocation in infants with ALL was placed at 85% [9]. The t(4;11) ALL is particularly resistant to conventional chemotherapies upon relapse, necessitating the evaluation of alternative therapeutics. We have previously shown that resveratrol can effectively induce apoptotic cell death in cell lines that were established from patients with high-risk, B-lineage acute lymphoblastic leukemia (ALL) carrying the t(4;11)(q21;q23) chromosomal translocation, as well as other ALL lines without the translocation [4]. Apoptosis was induced in these cells through a mitochondnal/caspase-9 pathway that was independent of CD95. In the current study, we utilized two modulators of MPTP opening to determine if resveratrol-induced apoptosis of the ALL cells could be enhanced. Cyclosporin A binds to cyclophilin D and blocks MPTP opening [10,11]. PK11195, a ligand of PBR, promotes the opening of the pore [12]. Both CsA and PK11195 have been reported to induce mitochondrial dysfunction and apoptosis in leukemic cells [13–15]. Since transient opening of the MPTP helps to maintain the $\Delta\Psi mt$, we hypothesized that perturbation of the MPTP with CsA or PK11195, by either blocking or promoting pore opening, would enhance resveratrol-mediated mitochondrial membrane depolarization leading to apoptosis in ALL-derived cells. We found that CsA or PK11195 pre-treatment could significantly enhance resveratrol-mediated loss of $\Delta\Psi mt$ and apoptotic cell death in t(4;11) and other ALL-derived cell lines. These data suggest that resveratrol in combination with other agents that disrupt mitochondrial homeostasis may be useful for the treatment of ALL.

2. Materials and methods

2.1. Reagents

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) and PK11195 (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl propyl)-3-isoquinolinecarboxamide) were purchased from Sigma (St Louis, MO). Resveratrol stock solutions were dissolved in methanol and PK11195 stocks were dissolved in dimethylsulfoxide (DMSO, Sigma) before use. Cyclosporin A (CsA) was purchased from EMD Biosciences (San Diego, CA) and concentrated stock solutions were dissolved in DMSO. JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) was purchased from Molecular Probes (Eugene, OR) and stock solutions were prepared in DMSO. Alexa Fluor 488-conjugated annexin V and propidium iodide (PI) were obtained from Molecular Probes and Sigma, respectively.

2.2. Cell culture

SEM and RS4;11 cell lines were established from patients diagnosed with pre-B cell acute

lymphoblastic leukemia (ALL) containing the chromosomal translocation t(4;11)(q21;q23) [16,17]. The presence of the t(4;11) translocation has been confirmed in these cell lines by polymerase chain reaction (PCR) as previously described [18]. REH (pre-B cell ALL without the translocation) and Jurkat T-cell ALL lines were obtained from American Type Culture Collection (Manassas, VA). All cell lines were maintained at 37 °C, 5% CO_2 in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma), 50 IU/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate, and 2 mM L-glutamine (Invitrogen). For each experiment, the cells were split to a density of 0.5×10^6/ml before treatment. As a control for the dissolving medium used for each chemical, an equivalent amount of the specific diluent was always added to a control cell population in every experiment (designated control cells).

2.3. Analysis of mitochondrial membrane potential and apoptotic cell death

To measure apoptotic cell death, cells were stained with Alexa Fluor 488-conjugated annexin V according to the manufacturer's protocol. Propidium iodide (PI) diluted in PBS was added to differentiate between early apoptosis and late apoptosis/necrosis. JC-1 dye was used to measure changes in mitochondrial membrane potential as previously described [4]. For dose response analyses, cells were pre-treated with different concentrations of CsA or PK11195 for 30 min at 37 °C and then incubated with either methanol as a control or resveratrol at a final concentration of 50 μM. The 30 min pre-incubation period was used based on preliminary experiments with several timepoints. Apoptosis and depolarization of mitochondrial membranes by resveratrol has been previously observed by 24 h after treatment [4]. Therefore, after 24 h, the cells were stained to measure apoptosis or mitochondrial membrane depolarization. Fluorescence analyses were performed on a FACSCanto fluorescence-activated cell sorter (FACS) using BD FACSDiva software, vs. 4.0 (Becton-Dickinson, San Jose, CA). All analyses of whole cells were performed using appropriate scatter gates to exclude cellular debris and aggregated cells. Ten thousand events were collected for each sample stained with annexin V/PI or JC-1.

2.4. Multidrug resistance and intracellular resveratrol retention

Multidrug resistance was measured in the cells using the Vybrant™ Multidrug Resistance Assay kit containing the fluorescent probe calcein from Molecular Probes following the manufacturer's protocol with modification. The cells (0.5×10^6) were untreated or pre-treated with a final concentration of 25 μg/ml CsA or 25 μg/ml verapamil for 15 min at 37 °C and then stained with 0.025 M calcein acetoxymethyl ester (AM) for another 15 min at 37 °C. Cells were then washed in complete medium and allowed to incubate for an additional 15 min at 37 °C before washing with PBS and analysis by FACS to determine loss of fluorescent signal.

The presence of resveratrol inside the cells was analyzed by detection of its autofluorescence spectrum using a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA). Cells were untreated or pre-treated with CsA or PK11195 for 30 min at 37 °C at a final concentration of 10 and 100 μM, respectively. Resveratrol was added at a final concentration of 50 μM and the cells were incubated 1 and 24 h at 37 °C before analysis. After washing in PBS, cells were resuspended in PBS at a concentration of 10^6 cells per ml and transferred to cuvettes containing stir bars. Cuvettes were placed in the multi-cell holder with constant stirring of cells and block temperature was set at 4 °C. The scan mode program in the Cary Eclipse analytical software vs. 1.1 was used with an excitation wavelength of 322 nm. Fluorescence of resveratrol was scanned between 350 and 500 nm and peak emission was recorded at approximately 390 nm. A standard curve was generated with different concentrations of resveratrol in PBS for estimates of the intracellular levels of resveratrol for each cell line.

2.5. Statistical analysis

All statistical analyses were performed with GraphPad software (GraphPad Software, Inc., San Diego, CA, USA) and data were displayed as arithmetic means ± standard error (SE). *P*-values

were obtained using two-tailed *t*-tests with a confidence interval of 95% for evaluation of the significance of differences between treatment groups.

3. Results

3.1. CsA and PK11195 enhance resveratrol-mediated apoptotic cell death

We have previously shown that resveratrol disrupts mitochondrial membrane potential resulting in activation of caspase-9 and subsequent apoptotic cell death in several leukemia-derived cell lines [4]. The MPTP is important for maintaining mitochondrial membrane homeostasis and opening of the MPTP is a central event during the induction of apoptosis. Cyclosporin A (CsA) binds to cyclophilin D and inhibits pore opening [10,11]. However, the benzodiazepine receptor ligand PK11195 promotes opening of the MPTP [12]. To determine whether modulating the opening and closing of the MPTP would alter resveratrol-induced apoptosis, leukemia lines

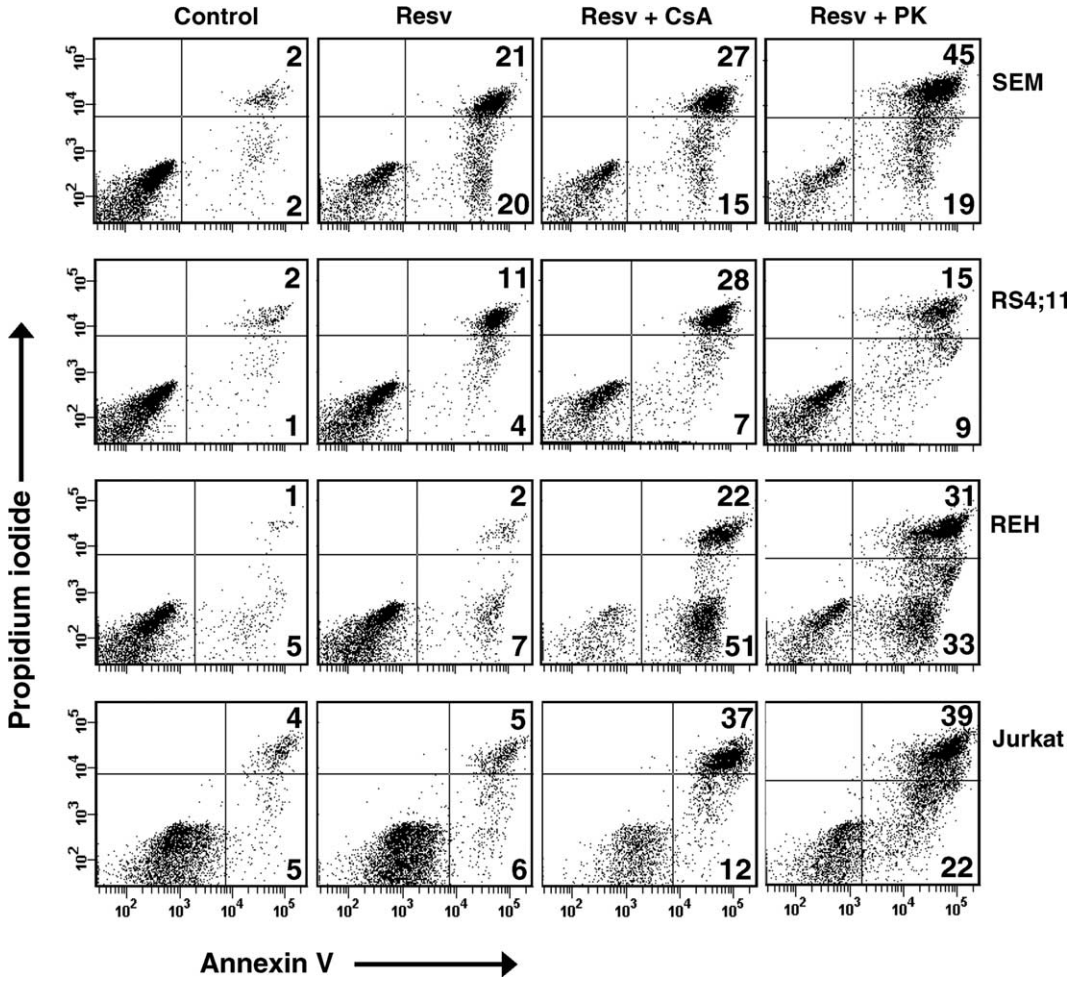

Fig. 1. CsA and PK11195 enhance resveratrol-mediated apoptosis. Cells were pre-treated with 10 μM CsA and 100 μM PK11195 for 30 min prior to the addition 50 μM resveratrol. After 24 h, the cells were stained with annexin V and PI and analyzed by FACS. The numbers at the bottom right quadrant of each dot plot represent the percentage of cells in early apoptosis (annexin V-positive, PI-negative). Numbers at the top right quadrant represent the percentage of cells in late apoptosis and/or secondary necrosis (annexin V positive, PI-positive). The data are representative of three separate experiments.

representing both B-lineage and T-lineage ALLs were used, including the two lines SEM and RS4;11 that were established from patients with high-risk t(4;11) ALL. The cells were pre-incubated with either vehicle, 10 μM CsA, or 100 μM PK11195 for 30 min and then treated with resveratrol for 24 h before staining with annexin V and propidium iodide (PI) [19,20]. PI, which does not enter cells with intact membranes, was used to differentiate between early apoptotic (annexin V-positive) and late apoptotic/necrotic cells (annexin V/PI double positive). Previous results from this laboratory indicated that apoptotic cell death could be clearly observed by 24 h after treatment with resveratrol alone. All four leukemia lines displayed populations of early apoptotic cells that stained only with the annexin after treatment with resveratrol alone, CsA plus resveratrol, and PK11195 plus resveratrol (Fig. 1, bottom right quadrant). CsA and PK11195 alone also displayed apoptotic populations (data not shown). Cells staining for both annexin V and PI were present (Fig. 1, top right quadrant) and represented later stages of apoptosis and cells undergoing rapid secondary necrosis in culture. These data showed that pre-incubating the cells with CsA or PK11195 induced apoptotic cell death and not primary necrosis when combined with resveratrol.

Cells were pre-treated with different concentrations of CsA and PK11195 in the presence or absence of 50 μM resveratrol to determine whether the increase in apoptosis was dose-responsive (Fig. 2). CsA pre-treatment substantially enhanced resveratrol-mediated cell death in RS4;11, REH, and Jurkat cells. In RS4;11, one of the t(4;11) ALL-derived lines, CsA pre-treatment increased death from 17.3±1.8% for resveratrol alone to 24.4±1.7% for 5 μM CsA plus resveratrol and up to 39.9±4.7% when treated with 10 μM CsA plus resveratrol ($P<0.05$). Cell death increased in REH cells from 10.5±2.2% for resveratrol alone up to 76.6± .6% when treated with 10 μM CsA plus resveratrol ($P<0.05$). And cell death increased from 10.3±1.8% for resveratrol alone to 45.5±6.1% for Jurkat cells treated with both 10 μM CsA and resveratrol ($P<0.05$). Increases in resveratrol-mediated death observed for the t(4;11) line SEM pre-treated with 5 and 10 μM CsA were statistically significant compared to cells treated with resveratrol alone, but the increase was not large (38.5±1.0 for resveratrol alone up to 47.7±2.6 and 43.4±1.0 for 5 and 10 μM CsA pre-treatment, respectively, $P<0.05$). CsA alone at a concentration of 10 μM induced apoptosis in SEM cells similar to the level observed for CsA plus resveratrol. However, pre-treatment of SEM cells with 100 μM PK11195 increased cell death from 40.2±1.8% for resveratrol alone up to 61.0±2.1% ($P<0.05$). RS4;11 did not show an increase in apoptotic cell death when pre-treated with PK11195 at the concentrations used.

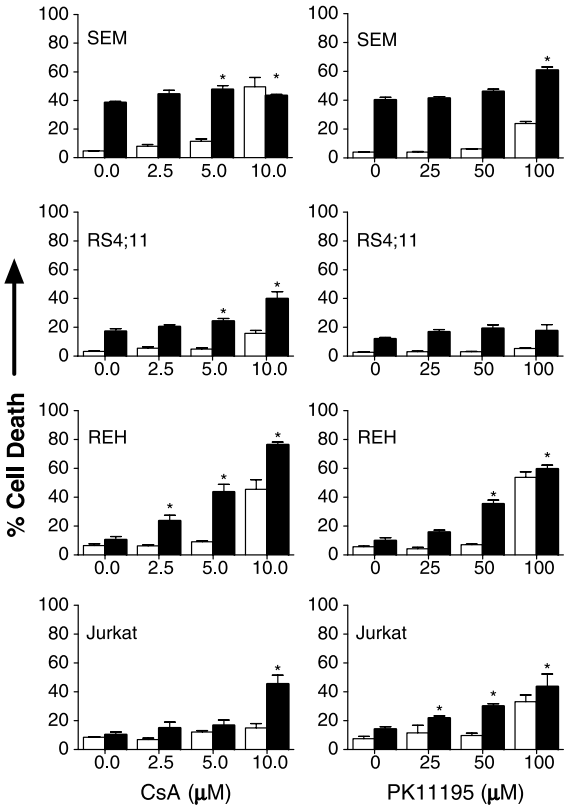

Fig. 2. Induction of cell death in response to different doses of CsA and PK11195. Cells were pre-treated with 0, 2.5, 5, and 10 μM CsA or 0, 25, 50, and 100 μM PK11195 for 30 min prior to addition of 50 μM resveratrol. After 24 h, cells were stained with annexin V and PI and analyzed by FACS for the percentage of cell death, which included both early and late apoptotic cells. White bars indicate cells treated with CsA or PK11195 alone. Black bars represent cells treated with CsA or PK11195 plus resveratrol. The data presented represent the mean of the percentage of cell death± SE from three separate experiments. Statistically significant differences ($P<0.05$) between cells treated with resveratrol alone and those treated with CsA or PK11195 plus resveratrol are indicated by the asterisk.

Apoptosis was significantly enhanced in REH and Jurkat ALL cells by PK11195 as well as CsA. Increases in cell death were from $9.9 \pm 1.9\%$ for resveratrol alone up to $59.7 \pm 2.5\%$ for REH and $14.2 \pm 1.6\%$ up to 43.7 ± 8.7 for Jurkat cells when pre-treated with 100 μM PK11195 ($P < 0.05$). REH cells also showed significant sensitivity to apoptosis when treated with the highest doses of CsA and PK11195 without addition of resveratrol. These results show that either CsA or PK11195 can enhance resveratrol-mediated apoptosis in the ALL-derived cell lines.

3.2. CsA and PK11195 enhance resveratrol-mediated mitochondrial membrane depolarization

Because we observed an enhancement of resveratrol-induced cell death by pre-treatment with CsA and PK11195, we next determined whether these agents were acting at the mitochondrial level to increase depolarization of the mitochondrial membranes. Cells were pre-incubated with CsA or PK11195 for 30 min and then treated with resveratrol for 24 h before staining with JC-1. JC-1 is a mitochondrial-selective dye and forms aggregates in normal polarized mitochondria that result in a green orange emission of 590 nm after excitation at 490 nm [21]. Upon depolarization of the mitochondrial membrane, JC-1 forms monomers that emit only green fluorescence at 527 nm. Fig. 3 shows a clear increase in the percentage of cells that emitted only green fluorescence after resveratrol treatment (bottom right quadrants). CsA enhanced resveratrol-induced mitochondrial depolarization in RS4;11, REH, and Jurkat cells, but not in SEM cells. PK11195 pre-treatment increased the percentage of cells with depolarized mitochondria above that observed for resveratrol alone in SEM, REH, and Jurkat cells (Fig. 3), but not in RS4;11, in agreement with the

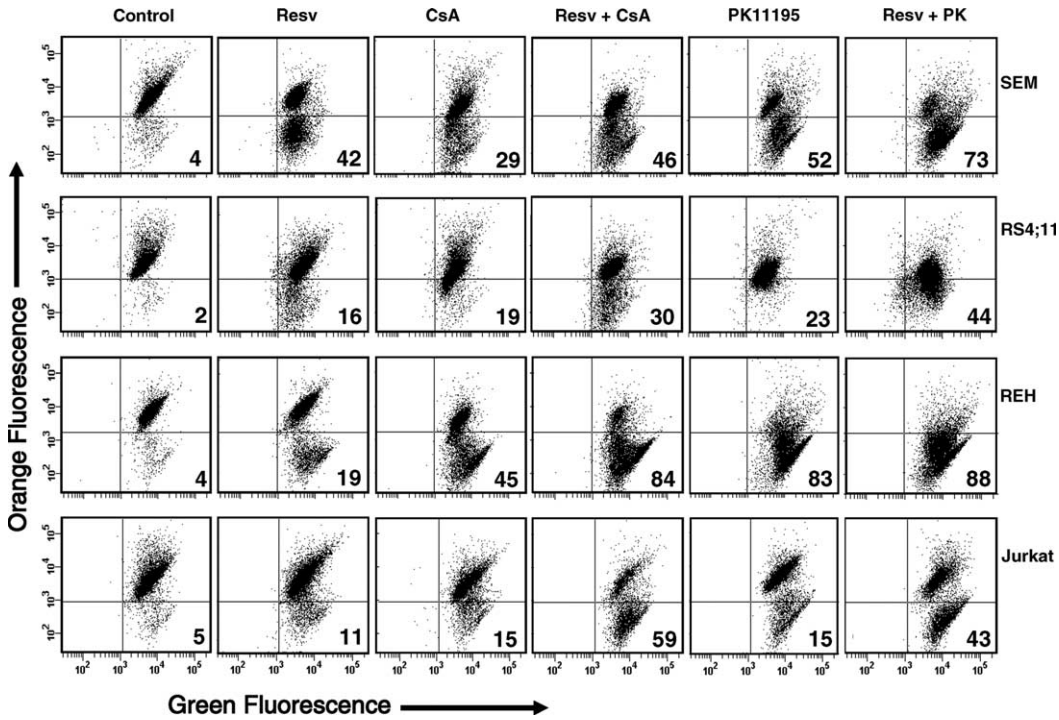

Fig. 3. CsA and PK11195 increase resveratrol-mediated depolarization of mitochondrial membranes. The cells were treated as described for Fig. 1. After 24 h, the cells were stained with the mitochondria selective dye JC-1 and analyzed by FACS. Cells with normal polarized mitochondrial membranes emit green-orange fluorescence (top right quadrant). The number in the bottom right quadrant of each dot plot represents the percentage of cells that emit only green fluorescence indicating loss of $\Delta\Psi mt$. The data are representative of three separate experiments.

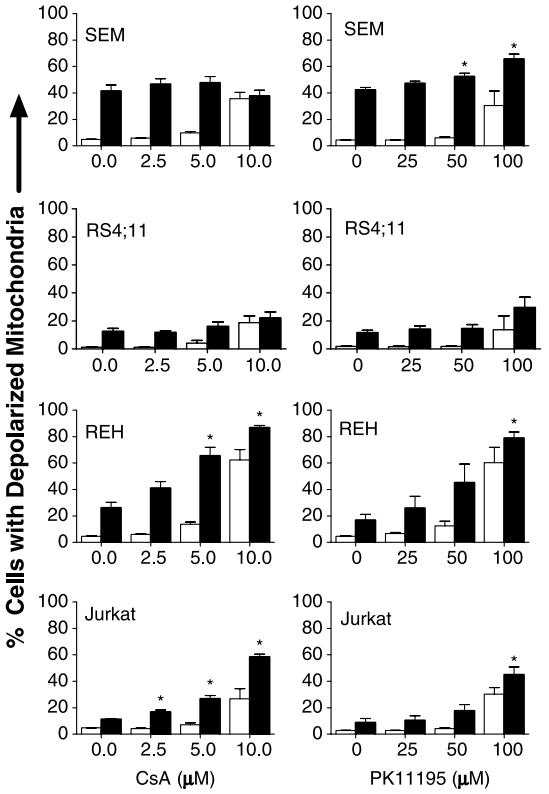

Fig. 4. Loss of $\Delta\Psi mt$ in response to different doses of CsA and PK11195. Cells were treated as described in Fig. 3. After 24 h, cells were stained with the mitochondria-selective dye JC-1 and analyzed by FACS for the percentage of cells with depolarized mitochondrial membranes. White bars indicate cells treated with CsA or PK11195 alone. Black bars represent cells treated with CsA or PK11195 plus resveratrol. The data presented represent the mean of the percentage of cell death ± SE from three separate experiments. Statistically significant differences ($P < 0.05$) between cells treated with resveratrol alone and those treated with CsA or PK11195 plus resveratrol are indicated by the asterisk.

annexin/PI staining for apoptosis (Figs. 1 and 2). Fig. 4 shows the dose-responsiveness of resveratrol-induced mitochondrial depolarization when cells were pre-treated with different concentrations of CsA and PK11195. The percentage of cells with depolarized membranes was similar to the percentage of cells staining with annexin V/PI in the presence of resveratrol and the indicated concentrations of CsA and PK11195. These results show that CsA and PK11195 do act at the mitochondrial level to significantly enhance resveratrol-mediated depolarization of mitochondrial membranes that leads to apoptotic cell death.

3.3. Multidrug resistance

CsA and PK11195 have both been shown to inhibit the cytosolic membrane transporter system responsible for efflux of drugs in acute myeloid leukemia cells, and, thereby, sensitized these cells to apoptotic effects of the chemotherapeutic agents [22,23]. Therefore, it was possible that one of the mechanisms by which CsA and PK11195 could be enhancing apoptosis in the leukemia cells was by inhibiting the transport of resveratrol out of the cell through these transport systems. To determine whether CsA and PK11195 were enhancing resveratrol-mediated apoptosis by altering the cytoplasmic membrane transporters, the cells were tested for multidrug resistance efflux. Cells were stained with calcein AM, a nonfluorescent, lipid soluble dye that converts to a highly fluorescent dye upon intracellular cleavage of the ester bonds. Calcein is a substrate for both the P-glycoprotein (Pgp) encoded by the multidrug resistance 1 gene (MDR1) and the multidrug resistance protein (MRP) encoded by the MDR2 gene [24,25]. Staining of the ALL cells with calcein in the presence or absence of the Pgp inhibitors CsA and verapamil showed that SEM, RS4;11, and REH cells do not have an active drug efflux system (Fig. 5). CsA and verapamil are the standard drugs used for testing multidrug resistance efflux, and since we observed no active drug efflux using these agents, PK11195 was not tested in these experiments. Untreated Jurkat cells showed only a slight decrease in fluorescence compared to cells treated with CsA or verapamil (black histogram). However, this change was not statistically significant. Therefore, calcein fluorescence was essentially the same with or without CsA and verapamil for all four lines. CsA and verapamil are inhibitors of the MDR1 gene product Pgp. To ensure that the cells did not have active MRP, cells were stained with calcein AM as described, washed, and placed on ice. The fluorescence intensity was compared to the cells that were incubated a further 15 min at 37 °C after staining. No difference in fluorescence intensities were observed between cells held on ice and those that were incubated for the full period (data not shown), indicating that there was no

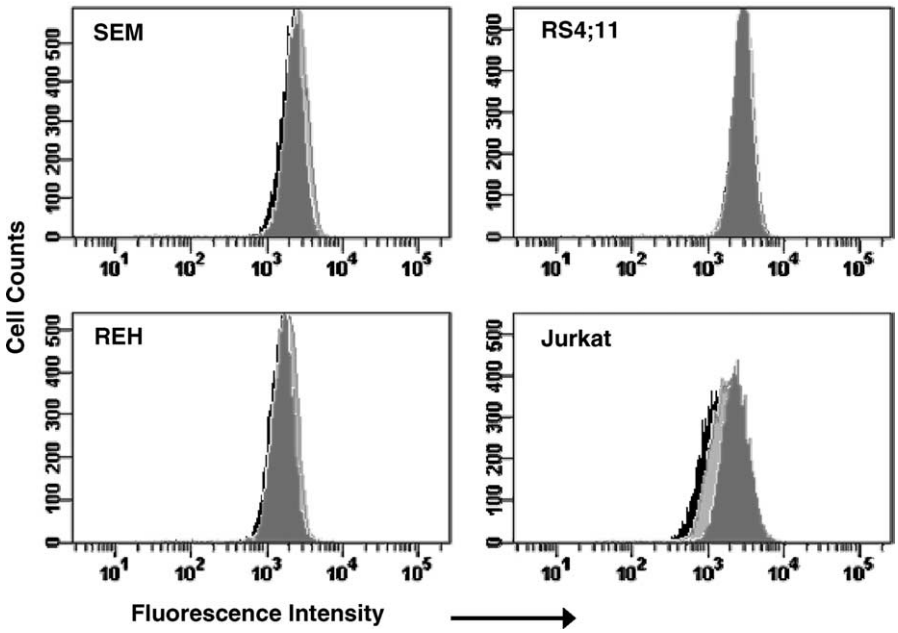

Fig. 5. Multidrug resistance in the leukemia-derived cells. Cells were untreated (black histogram) or pre-treated with a final concentration of 25 μg/ml CsA (light gray histogram) or 25 μg/ml verapamil (dark gray histogram) for 15 min at 37 °C and then stained with 0.025 μM of the fluorescent dye calcein AM. Cells were then washed and allowed to incubate for an additional 15 min at 37 °C before washing and analysis by FACS for loss of fluorescence intensity due to efflux of the dye. The data are representative of three separate experiments.

active transport of the calcein out of the leukemia cells by either Pgp or MRP mechanisms. These data coupled with the increased mitochondrial membrane depolarization observed (Figs. 3 and 4) strongly suggest that both CsA and PK11195 are acting at the level of the MPTP to enhance resveratol-induced apoptosis in the leukemia cells.

3.4. Intracellular retention of resveratrol

A second method was used to determine whether CsA and PK11195 could be acting by inhibiting drug efflux mechanisms and inducing apoptosis by increasing the intracellular levels of resveratrol. Resveratrol autofluorescence was used to directly detect relative concentrations of the substance inside the leukemia cells after treatment for 1 h and after 24 h. Using a Cary Eclipse fluorescence spectrophotometer, the excitation maximum for resveratrol in PBS was determined to be 322 nm. Cells were pre-treated with 10 μM CsA or 100 μM PK11195 for 30 min and then incubated with 50 μM resveratrol for 1 and 24 h. Emission spectra were scanned between the range of 350 and 500 nm using 322 nm excitation. CsA and PK11195 in solution and cells incubated with CsA and PK11195 alone were also measured to determine whether these drugs autofluoresced at these excitation and emission wavelengths. No autofluorescence was observed with these compounds (data not shown). Pre-treatment with CsA and PK11195 did not alter the levels of resveratrol inside SEM, RS4;11, and REH cells after 1 h incubation as determined by the intensity of fluorescence emission (Fig. 6). Surprisingly, pre-treatment with both CsA and PK11195 decreased the fluorescent signal of resveratrol in Jurkat cells. A standard curve with different concentrations of resveratrol in PBS was used to estimate the average content of resveratrol in each cell. The concentration of resveratrol was decreased by approximately 60% in Jurkat cells treated with resveratrol and CsA or PK11195 compared to resveratrol alone (Table 1). Since we did not observe a significant cytoplasmic membrane efflux operating in these leukemia cells by 1 h (Fig. 5), these data

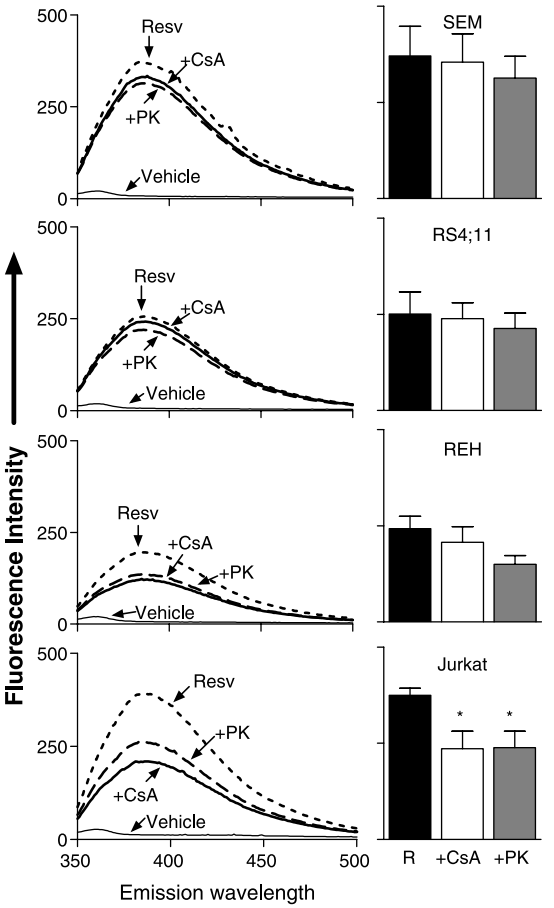

Fig. 6. Intracellular retention of resveratrol in the presence of CsA and PK11195. Cells were pre-treated with 10 μM CsA or 100 μM PK11195 for 30 min at 37 °C prior to the addition of 50 μM resveratrol. After an addition 1 h incubation at 37 °C, the cells were washed and fluorescence of resveratrol was measured by spectrophotometry using Cary Eclipse analytical software vs. 1.1. Analyses were performed using an excitation wavelength of 322 nm for resveratrol with emission spectra recorded between wavelengths of 350–500 nm (left side of Fig). The average results ± SE of three independent experiments for SEM, RS4;11, and Jurkat and five independent experiments for REH cells are shown on the right side. Statistically, significant differences between cells treated with resveratrol alone and those treated with CsA or PK11195 plus resveratrol are indicated by the asterisk.

suggest that CsA or PK11195 may be limiting entry of resveratrol into Jurkat cells. Analysis of resveratrol fluorescence after 24 h showed a variable loss of resveratrol from the cells, likely due to leakage of the drug from apoptotic and secondary necrotic cells in the culture (data not shown).

4. Discussion

Resveratrol has shown promise as a novel chemotherapeutic agent against a variety of cancers. Resveratrol treatment has been shown to suppress proliferation in cancer cells by decreasing the expression or activities of cell cycle regulators, such as the cyclin-dependent kinases CDK4 and Cdc2 and, subsequently decreasing the activities of the cyclin-CDK complexes which drive the cell to replicate (reviewed in Ref. [2]). Other cell cycle regulatory proteins that are inhibited or activated by treatment with resveratrol include retinoblastoma protein, $p21^{Cip1/WAF1}$, and the transcription factors p53 and E2F. Resveratrol was shown to directly destroy the tyrosyl radical on the R2 subunit of ribonucleotide reductase, the enzyme responsible for the conversion of ribonucleotides to 2′-deoxyribonucleotides which provides the precursor molecules necessary for both DNA synthesis and repair [26]. Inhibition of this enzyme activity results in arrest of the cell in the G1 phase of the cell cycle. In *ex vivo* purging experiments, it was shown that, although resveratrol could inhibit the growth of both leukemic and normal bone marrow progenitor cells, growth arrest of the normal hematopoietic progenitors was achieved at higher resveratrol concentrations [27]. Resveratrol

Table 1
Approximate fmoles of resveratrol per cell after 1 h in the presence or absence of 10 μM CsA or 100 μM PK11195. A standard curve was generated with different concentrations of resveratrol in PBS to estimate the content of resveratrol per cell. Resveratrol autofluorescence was measured for each concentration by fluorescence spectrophotometry

Cell line	Treatment	Resveratrol content per cell (fmoles/cell ± SE)
SEM	Resveratrol	20.6 ± 10.4
	CsA + Resveratrol	18.2 ± 9.7
	PK11195 + Resveratrol	11.8 ± 4.6
RS4;11	Resveratrol	7.5 ± 2.9
	CsA + Resveratrol	6.3 ± 1.8
	PK11195 + Resveratrol	5.2 ± 1.4
REH	Resveratrol	6.7 ± 2.4
	CsA + Resveratrol	5.5 ± 2.3
	PK11195 + Resveratrol	3.1 ± 0.7
Jurkat	Resveratrol	16.0 ± 2.0
	CsA + Resveratrol	6.3 ± 2.3*
	PK11195 + Resveratrol	6.2 ± 1.7*

*Statistically significant difference compared to cells treated with resveratrol alone, $P < 0.05$.

inhibited growth of normal progenitors at an IC_{50} of 59 μM versus 18–34 μM for the leukemic cells. Furthermore, in these experiments, it was shown that the normal progenitor cells significantly recovered their ability to engraft in vivo after an *ex vivo* treatment with 80 μM resveratrol, whereas the anti-leukemic effect was highly irreversible after treatment of the cells.

Induction of apoptosis by resveratrol occurs through CD95- and mitochondrial-mediated pathways depending on the cancer cell type. Mitochondrial-mediated apoptosis may be induced by resveratrol through direct inhibitory action on the F0/F1-ATPase proton pump of the mitochondrial inner membrane [3]. Resveratrol decreases the expression of anti-apoptotic Bcl-2 proteins in a number of different cancers [2]. Both anti-apoptotic and pro-apoptotic Bcl-2 family members play an important role in regulating the integrity of the mitochondrial membrane [28]. The anti-apoptotic proteins Bcl-2 and Bcl-XL, as well as the pro-apoptotic proteins Bax and Bak, interact with the ANT that is part of the MPTP [29–31].

Tinhofer et al. [5] showed that resveratrol treatment induced swelling of mitochondria that was blocked by CsA, indicating that resveratrol itself induced an opening of the MPTP. MPTP opening can occur under conditions of oxidative stress and these authors showed that disruption of mitochondrial membranes by resveratrol was accompanied by the generation of reactive oxygen species (ROS). As a by-product of electron transport uncoupling, ROS can further induce and maintain a collapse of $\Delta\Psi mt$ leading to cellular damage through oxidation of lipids and proteins resulting in apoptotic cell death. It should be noted that Tinhofer et al. [5] observed CsA inhibition of mitochondrial swelling during a 3–5 min period after addition of reagents. Previous reports have indicated that CsA only transiently blocks opening of the MPTP [32,33]. Therefore, CsA may be interacting with other proteins in the leukemia-derived cells to enhance resveratrol-mediated apoptosis.

CsA and PK11195 have both been utilized to inhibit MDR efflux systems in leukemia cells resulting in sensitization of these cells to chemotherapeutic agents [22–34]. In our experiments, we observed little or no MDR efflux occurring in the leukemia-derived cells using the MDR substrate calcein, suggesting that both CsA and PK11195 were acting on the mitochondria to produce depolarization and apoptotic cell death. In the t(4;11) ALL-derived lines SEM and RS4;11 and in REH cells, there was no difference in intracellular resveratrol content in the presence or absence of CsA and PK11195. However, Jurkat cells treated with resveratrol plus CsA or PK11195 showed a decrease in intracellular resveratrol content. It is unclear whether CsA or PK11195 was able to inhibit the entry of resveratrol into Jurkat cells. However, in these leukemia cells, CsA or PK11195 was able to significantly enhance resveratrol-mediated cell death (Fig. 3), suggesting that even with a lowered intracellular concentration of resveratrol, the combination of agents is still more effective in the induction of apoptosis than resveratrol alone.

B-lineage ALLs are commonly resistant to CD95-mediated apoptosis [35], but appear to be sensitive to agents that act to through mitochondria. We have previously shown that SEM, RS4;11, and REH cells are resistant to CD95-signaling mediated by CD95 ligand and this resistance is at least partially due to terminal glycosylation of CD95 in these cells [4,18,36]. Although resveratrol has been shown to act by stimulating CD95-signaling in some cancer cells, we have observed that induction of apoptosis in the B-ALL lines by resveratrol occurs exclusively through disruption of mitochondrial homeostasis [4]. These results led us to continue our investigations of the role mitochondria play in resveratrol-induced apoptosis and evaluate agents that would act in concert to enhance the mitochondrial-mediated apoptosis in these leukemia cells. Resveratrol and MPTP modulators, such as CsA and PK11195, act directly on specific protein complexes in the mitochondria of these leukemia-derived cells which results in loss of $\Delta\Psi mt$, making these agents potentially useful as combinatorial chemotherapeutics. PK11195 is well-tolerated in humans as a single agent [37,38]. Although CsA can cause significant toxicities in humans, particularly renal toxicity [22], this compound has been previously proposed as potential chemotherapeutic agent for treatment of ALL [15]. Furthermore, the CsA analog PSC-833, which is well tolerated and also induces mitochondrial dysfunction, was recently studied in phase I clinical trials in patients with refractory acute myeloid

leukemia [13,39]. The results presented in this study suggest that targeting mitochondrial function with modulators of MPTP and resveratrol to produce apoptosis may be effective as an alternative and novel chemotherapeutic strategy in the treatment of ALL, particularly the high-risk, t(4;11) ALL. Future investigations using clonogenic assays with multiple drug treatments, as well as animal experiments using the NOD/SCID mouse model for ALL [40,41] will be necessary to further evaluate the efficacy of these agents against ALL.

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